

# Physicochemical characterization of six monoclonal cryoimmunoglobulins: Possible basis for cold-dependent insolubility

(cryoglobulin/circular dichroism/fluorescence/analytical gel filtration/solute variation)

C. R. MIDDAGH\*, B. GERBER-JENSON\*, A. HURVITZ†, A. PALUSZEK\*, C. SCHEFFEL\*,  
AND G. W. LITMAN\*‡

\* Sloan-Kettering Institute for Cancer Research, Walker Laboratory, 145 Boston Post Road, Rye, New York 10580; and † Animal Medical Center, 510 East 62nd Street, New York, New York 10021

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**ABSTRACT** The physical and chemical properties of five human and one canine monoclonal cryoimmunoglobulin have been compared. By many criteria, the proteins cannot be distinguished from the noncryoglobulin reference proteins analyzed in parallel; however, certain hydrodynamic and spectroscopic properties of the proteins indicate that cryoimmunoglobulins differ in tertiary structure relative to their cold-soluble counterparts. These differences seem to favor low-temperature-induced association between cryoglobulin molecules as an immediate consequence of increased intermolecular ionic or van der Waals forces. No evidence was found for the formation of cold-dependent antigen-antibody complexes or the ubiquitous presence of low-temperature-dependent conformation changes as a component of cryoprecipitation. Rather, the anomalous solution behavior of monoclonal cryoimmunoglobulins can be considered a direct result of the individual solubility properties of these proteins.

Cryoimmunoglobulins are immunoglobulins that undergo precipitation as the temperature of the plasma is decreased below 37°. Frequently, the solubility difference between cold-soluble and insoluble immunoglobulins is greater than 1000-fold at low temperatures. The occurrence of a cryoprotein was first noted by Wintrobe and Buell (1) in a patient with clinical and pathological features consistent with multiple myeloma. Lerner *et al.* (2) first classified the cryoprotein of another patient with multiple myeloma as a gamma globulin. Cryoimmunoglobulins now are recognized to be associated with various lymphoproliferative disorders and both acute and chronic infectious diseases. Depending on the relative concentration, temperature-dependent solubility behavior, and protein type(s) involved, the presence of these proteins can result in a wide range of vascular, renal, and neurological complications (3–6). Although the clinical significance of cryoglobulins is clearly recognized, little is known concerning the structural basis for the anomalous temperature-dependent solubility exhibited by these proteins.

Recently, we examined the monoclonal IgM cryoimmunoglobulin McE by using spectroscopic (7, 8), hydrodynamic (8), solute variation (9, 10), and immunochemical (11) methods. This report describes extension of these studies to include five additional proteins that can unequivocally be categorized as monoclonal cryoimmunoglobulins and to compare the structural and cold-dependent insolubility properties of these proteins to each other and, where applicable, to reference monoclonal immunoglobulins lacking cryoglobulin tendencies. Evidence is presented that certain structural features distinguish some cryoglobulins from noncryoglobulin proteins; however, there appears to be no single structural anomaly common to all monoclonal cryoimmunoglobulins.

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## MATERIALS AND METHODS

**Isolation and Characterization of Immunoglobulins and Immunoglobulin Fragments.** The procedures used in the isolation and characterization of the monoclonal cryoimmunoglobulins were as described (11). Noncryoglobulin, polyclonal, and monoclonal IgMs were isolated by repeated euglobulin precipitation and gel filtration on agarose, and monoclonal IgGs were isolated by ion exchange chromatography on DEAE-Sephadex (Pharmacia). Procedures for the production and characterization of 7S IgM, (Fc)<sub>5</sub>μ, and Fabμ have been described (11). Pepsin Fab'₂ (12) and papain Fab plus Fc (13) were produced and isolated by the methods indicated.

**Analytical Procedures.** Automated NH₂-terminal amino acid sequencing was performed essentially as described (11). High-performance liquid chromatography (14) and back hydrolysis with HI to the free amino acid were used to confirm the identity of the phenylthiohydantoin amino acid derivatives. Subgroups were assigned by homology with prototype sequences (15). The assays of cryoprecipitation (11), including solute variation studies (10), and determination of amino acid composition were carried out as indicated (11). pI measurements were made with monomeric (noncryoprecipitating) subunits of IgM and low (subcryoprecipitating) concentrations of IgG on Ampholine slab gels (LKB). Linear pH gradients from 3.5 to 9.5 were consistently achieved. In order to ensure comparable sulfhydryl modification of the IgMs, quantitative radioalkylation ([¹⁴C]iodoacetamide, New England Nuclear) was performed. Molecular weight estimation (8, 11) and spectroscopic measurements (7) were performed as described. The values (mean ± SEM) of E<sub>1</sub><sup>1%</sup><sub>cm,280</sub> not reported previously were Pel = 12.2 ± 0.2, Gre = 12.1 ± 0.2, Mel = 11.9 ± 0.4, and Ger 13.5 ± 0.3 in 0.15 M NaCl (pH 7.0).

## RESULTS AND DISCUSSION

**Immunochemical Typing and Heavy and Light Chain Subgroups of Monoclonal Cryoimmunoglobulins.** The isolated cryoimmunoglobulins were typed by double-diffusion reaction using monospecific antisera to κ and λ light chains and μ, α, γ, δ, and ε heavy chains. All of the human proteins reacted with anti-κ but not with anti-λ and only with anti-μ or anti-γ but not both. Based on these observations, the cryoglobulins were classified as either IgM, κ (Gre, McE, Pel) or IgG, κ (Ger and Mow). The heavy chain subclass of both Ger and Mow was IgG1. The canine cryoglobulin Mel was previously classified as an IgM (16). Based on NH₂-terminal amino acid sequencing, the light chain variable region subgroups were found to be Pel, V<sub>κ</sub>I; Gre, McE, and Mow, V<sub>κ</sub>III; and Ger, V<sub>κ</sub>IV. The heavy

‡ To whom correspondence should be addressed.

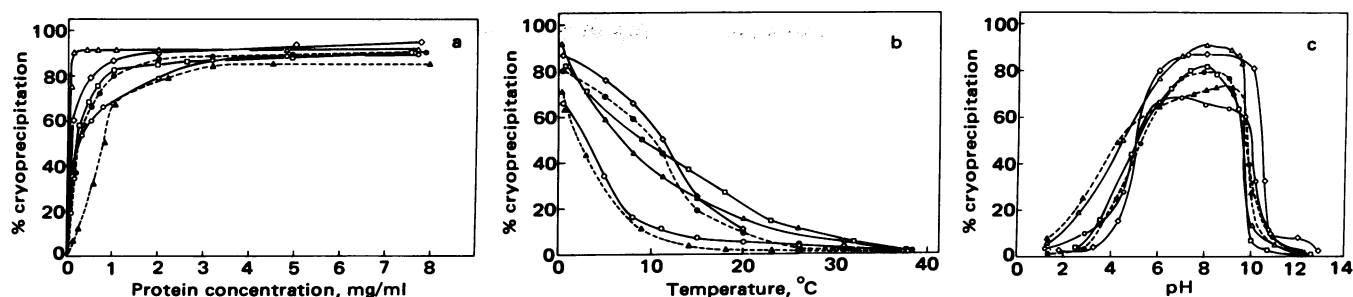


FIG. 1. Effect of protein concentration (a), temperature (b), and pH (c) on the cryoprecipitation of six monoclonal cryoimmunoglobulins. The protein concentration was 1 mg/ml throughout; 0.15 M Tris-HCl, pH 8.0, was used in a and b. Protein was dissolved in 0.15 M NaCl in c, and the pH of individual solutions was adjusted by addition of NaOH or HCl. Symbols: —, IgMs;  $\Delta$ , Mel;  $\diamond$ , McE;  $\square$ , Gre;  $\circ$ , Pel; ----, IgGs;  $\bullet$ , Ger;  $\blacktriangle$ , Mow.

chain sequences of Mow and Pel resembled prototypic  $V_{HIII}$  sequences.<sup>§</sup> The heavy chains of Ger, Gre, and McE were blocked; McE has previously been classified as  $V_{HV}$  (11).

**Effect of Protein Concentration, Temperature, and pH on Cryoprecipitation.** The effects of varying protein concentration, temperature, and pH on the cold-induced insolubility of the six monoclonal cryoimmunoglobulins are illustrated in Fig. 1a. Each of the proteins showed the loss of low-temperature insolubility at low protein concentrations predicted from simple solubility considerations, although quantitative differences were evident among the cryoglobulins. In general, however, the proteins did not appear to obey the Gibbs phase rule, because the amount of soluble protein was dependent on the total cryoglobulin concentration over a rather wide concentration range. A simple explanation for this atypical behavior suggests the presence of low-temperature-induced aggregates in equilibrium with the solid phase. This is supported by the marked curvature of the protein concentration-solubility plots at low protein concentration, an observation inconsistent with the theoretically predictable solubility behavior of homogeneous substances.

Differences between the cryoimmunoglobulins were also evident in the effect of temperature on their individual solubilities (Fig. 1b). Although each of the proteins appeared to be soluble at 38°, in every case, detectable insolubility began to occur below 20° (protein concentration = 2.5 mg/ml).

Each of the proteins exhibited a broad pH maximum for cryoprecipitation between pH 5.5 and 9.5 (Fig. 1c). There appeared to be a rather abrupt loss of cryoprecipitability between pH 9.5 and pH 10.5 and a more gradual abolition of the cold-induced insolubility between pH 3 and pH 5.5. The basic transition was in the region where tyrosine and lysine side chains would be expected to undergo deprotonization; the loss of cryoglobulin behavior in the acidic region most probably involved the deionization of aspartic and glutamic acid carboxyl groups. Preliminary spectroscopic studies indicated that the loss of cryoprecipitability at acidic pH is accompanied by protein structural transitions whereas the effect at basic pH appears to occur prior to any detectable conformational change(s).

**Effect of Solutes on Cryoprecipitation.** The effects of several classes of low molecular weight solutes upon the cold-induced insolubility of the cryoimmunoglobulins are presented in Fig. 2. These results can be summarized as follows. A non-chaotropic neutral salt (NaCl) inhibited the cryoprecipitation

of four of the proteins (Pel, Mel, Ger, and Mow) but appeared to have little effect other than a nonspecific Debye-Hückel charge-shielding interaction as manifested by a small enhancement of cryoprecipitation at low NaCl concentrations with two of the IgM cryoglobulins (McE and Gre). All of the proteins were inhibited by chaotropic salts (NaSCN,  $MgCl_2$ ) with varying degrees of effectiveness. Both urea and formamide also were potent inhibitors of cryoprecipitation at subdenaturing concentrations. The efficiency of urea as an inhibitor, however, was reduced by the substitution of alkyl groups onto the parent compound with the more hydrophobic solute (propylurea) a less-effective inhibitor than the ethyl-substituted agent. This effect was most noticeable with proteins McE, Pel, and Mel, less evident with Gre and Mow, and apparently absent with Ger. To a lesser extent, a similar pattern could be observed with alcohols. The increasing hydrophobicity of the alcohol was associated with an enhancement of cold-induced insolubility, particularly with McE and Pel at low alcohol concentrations. Sugars (galactose and ribose) also were found to inhibit cryoprecipitation, with the hexose being somewhat more effective than the pentose.

These observations can be interpreted in terms of the effect of these solutes upon the activity coefficients of model compounds (10). Based on both the data presented above and thermodynamic considerations, the following conclusions are possible: (i) the enhancement of cryoprecipitation observed with hydrophobically substituted solutes suggests that intermolecular hydrophobic interactions are not directly responsible for cryoprecipitation, and (ii) the inhibition of cryoprecipitation by the neutral salt NaCl observed with four of the six proteins suggests electrostatic (ionic) interactions as a major driving force in the cold-induced insolubility of some of the proteins. Because hydrogen bonding is unlikely to make a major contribution to stabilization of the cryoprecipitate (17), by elimination it would appear that van der Waals interactions may be involved in the cryoprecipitation of the remaining proteins (McE and Gre). Both of these proteins are of the IgM class in which the large surface areas and pentameric symmetry of these molecules might allow extensive van der Waals contacts.

An alternate interpretation of all of these studies, which must be considered, is that an unspecified combination of weak nonvalent interactions is responsible for the low-temperature insolubilization. This hypothesis is rather difficult to evaluate, however, because the solutes frequently have opposite effects upon the various types of weak interactions encountered in biologic systems.

**Cryoprecipitation Properties of Isolated Cryoimmunoglobulin Fragments and Subunits.** Each of the monoclonal IgM cryoimmunoglobulins was found to yield the characteristic 7S monomeric subunit after partial reduction with di-

<sup>§</sup> Protein Pel showed a Leu  $\rightarrow$  Val interchange at heavy chain position 4. To our knowledge, this variation has not been observed previously in other  $V_{HIII}$  sequences including proteins from various lower vertebrate species which have been interpreted as being homologous with the human  $V_{HIII}$  subgroup.

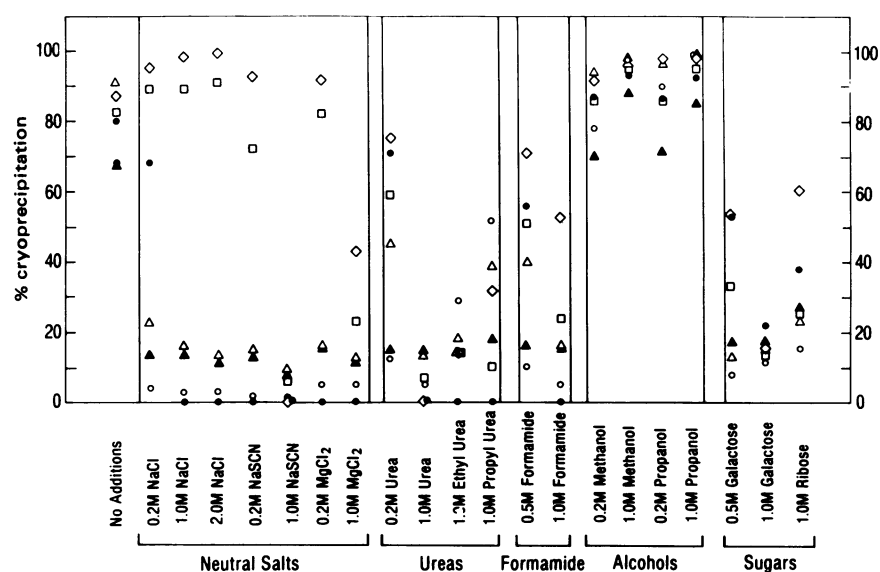


FIG. 2. Effect of solutes on the cryoprecipitation of six monoclonal cryoimmunoglobulins. All assays were performed at a protein concentration of 1 mg/ml in 0.15 Tris-HCl, pH 8.0. Values presented are an average of three determinations and the uncertainty of the values is 2–3%. Symbols as in Fig. 1.

thiothreitol. In addition, the three human monoclonal IgM cryoglobulins yielded the expected  $(Fc)_5\mu$  and  $Fab\mu$  fragments after digestion with trypsin at  $56^\circ$ , and the two IgG cryoglobulins yielded characteristic  $Fab'_2$  and  $Fab$  plus  $Fc$  fragments when digested with pepsin and papain, respectively. None of the subunits or fragments (or fragments in combination) at concentrations of 5 mg/ml (minimum) and in some cases exceeding 100 mg/ml in isotonic buffer were cryoprecipitable, nor were they capable of inhibiting cryoprecipitation of the parent protein. These results are inconsistent with the formation of cold-insoluble antibody-antigen complexes.

**Amino Acid Composition and Determination of pI.** Amino acid composition analyses of the heavy chains, light chains, and  $Fab\mu$ ,  $Fab$ ,  $(Fc)_5\mu$ , and  $Fc$  fragments failed, with the exception of the McE, to reveal differences between cryoglobulin and noncryoglobulin proteins (not illustrated). McE has significantly more tryptophan and less tyrosine in the  $Fd$  region of the heavy chain (11). The lack of difference between McE and proteins in this study extends to comparison of amino acid groups (e.g., aromatic, polar, nonpolar, hydroxylated, etc.). It should be noted, however, that the failure to distinguish asparagine from aspartic acid and glutamine from glutamic acid could be obscuring significant differences.

The pIs of the 7S subunits derived from McE and Pel (7S subunits of Gre are insoluble at  $37^\circ$ ) were found to be identical (5.4–5.9) and within the 4.9–5.3 to 6.9–7.6 range observed for the monoclonal and polyclonal IgM reference proteins. Similarly, the pIs of the IgGs, Ger, and Mow, (6.9–7.1) and (8.4–8.5) were within the range of the reference proteins; there appeared to be no obvious relationship between pI and cryoprecipitation, although two IgM proteins, McE and Pel, did have virtually indistinguishable isoelectric focusing behavior.

**Apparent Molecular Weights of Cryoglobulins and Their Fragments.** When the analytical gel filtration characteristics (under neutral aqueous conditions) of cryoglobulin and noncryoglobulin proteins and their subunits and fragments were compared, a striking difference was noted in three of the six proteins (Table 1). This difference was abolished under denaturing conditions and its origin (presumably in tertiary structure) is unclear; it may be due to an actual shape difference in the  $Fab$  or it may arise from some type of interaction (or lack of it) between the cryoglobulin and the sieving matrix.

**Spectroscopic Studies.** The secondary and tertiary structures

of each of the cryoimmunoglobulins were examined by various spectroscopic techniques. The ultraviolet absorption spectra of the proteins (215–300 nm) with the exception of McE (7) were found to be remarkably similar. The circular dichroism spectra at  $37^\circ$  of the IgM and IgG cryoimmunoglobulins along with corresponding spectra of comparison cold-soluble immunoglobulins are illustrated in Fig. 3. In all cases, the circular-dichroism minimum was located at 217–218 nm, consistent with the predominance of  $\beta$ -type secondary structure. Two of the IgM proteins (Pel and McE) and one of the IgG cryoglobulins (Mow) had negative ellipticity minima values outside the range encountered for noncryoglobulin IgM and IgG proteins.

Table 1. Differences in gel filtration behavior between normal and cryoglobulin immunoglobulins and their fragments

Protein	Apparent molecular weight difference	
	Intact	$Fab(\mu$ or $\gamma)$
McE (IgM)	125,000	13,000*
Gre (IgM)	96,000	6,000*
Ger (IgG)	74,000	10,000†

Analytical gel filtration was performed at  $37 \pm 0.5^\circ$  on a  $2.4 \times 100$  cm column of Bio-Gel A1.5m in 0.15 M Tris-HCl/0.02%  $NaN_3$ , pH 8.0. For determination of molecular weight under denaturing conditions, analytical gel filtration on Sephadex G-150 in 6 M guanidine-HCl or polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate was used. Calculated differences were obtained by subtracting the observed molecular weight of the cryoglobulin or its fragment from the corresponding value of noncryoglobulin standards analyzed in parallel. Standard molecular weight values either have been reported previously (8) or are 104,000, 52,000, and 50,000 for  $Fab'_2$ ,  $Fab$ , and  $Fc$ , respectively. Error values have been previously established for this type of analytical gel filtration system (8) and, in addition, it should be noted that the values for  $Fab(\mu$  or  $\gamma)$  are inferred from co-chromatography of the individual proteins with radioiodinated markers. No difference was noted for  $(Fc)_5\mu$  or  $Fc$  under neutral aqueous or in 0.1% sodium dodecyl sulfate or for immunoglobulin heavy and light chains examined in 6 M guanidine-HCl. The observed differences for  $Fab(\mu$  or  $\gamma)$  were abolished under denaturing conditions.

\* The ~7S monomeric subunit of McE revealed a value of 30,000 (abolished in 6 M guanidine-HCl or 0.1% sodium dodecyl sulfate). Insolubility of the monomeric subunit of Gre precluded estimation of apparent molecular weight under neutral aqueous conditions.

† The  $Fab'_2$  fragment of Ger exhibited a value of 37,000 (abolished under denaturing conditions).

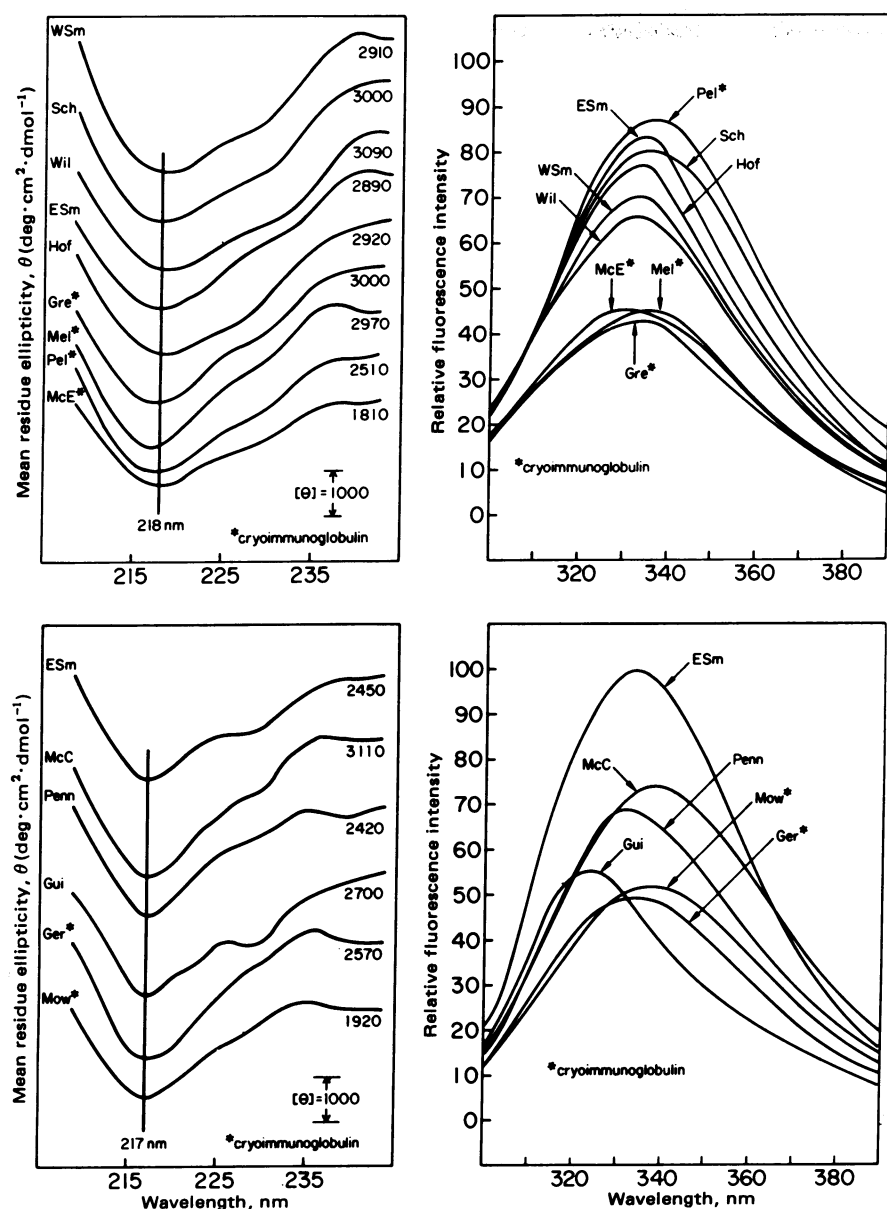


FIG. 3. Far UV circular dichroism spectra (Left) and intrinsic fluorescence emission spectra (Right) ( $\lambda_{ex} = 275$  nm) at 37° of cryoglobulin and noncryoglobulin proteins. The intensity of the ellipticity minima are given to the right of each circular dichroism spectrum. Spectra were obtained at a protein concentration of 0.08 mg/ml in 0.01 M sodium hydrogen phosphate/0.5 M NaCl, pH 7.0. (Upper) IgM proteins. The IgM protein designated ESm represents one element of a triclonal gammopathy IgM, IgC, and IgA,  $\kappa$ . (Lower) IgG proteins. Reference proteins Penn and McC were both classified as IgG<sub>1</sub>,  $\kappa$ .

Intrinsic fluorescence emission spectra of the same proteins at 37°C are also illustrated (Fig. 3). The emission peaks of three of the IgM cryoglobulins (Mel, Gre, and McE) were quenched relative to the noncryoglobulin proteins. Furthermore, both IgG cryoglobulins had quenched fluorescence emission, although one of the cold-soluble IgG proteins (Gui) also displayed a decreased intensity in comparison to the other three noncryoimmunoglobulin molecules probably as a direct consequence of its unusual tyrosine content (approximately 55% lower than normal). This relative decrease in emission intensity was also observed when the excitation wavelength was shifted from 275 nm (where both tyrosine and tryptophan should be excited) to 295 nm (which should produce emission almost exclusively from tryptophan), although the apparent quenching of cryoglobulin fluorescence was decreased to some extent. Because both the tyrosine and tryptophan compositions of the various proteins, with the exception of the McE and Gui proteins noted above, were found to vary by less than 10%, it is unlikely that the observed differences can be directly attributed to differences in the content of aromatic amino acids. The most probable

explanation for this decreased fluorescence is that cryoglobulin indole sidechains exist in an environment whose polarity differs significantly from that in cold-soluble immunoglobulins.

The possibility that the abnormal circular dichroism spectra of McE, Pel, and Mow are the result of an atypical secondary structure was investigated by both infrared (McE, Pel, and Mow) and Raman (McE and Pel) spectra (G. J. Thomas, personal communication) in <sup>2</sup>H<sub>2</sub>O films and in aqueous solution at 37°, respectively (not illustrated). As estimated by the position and relative intensity of the amide bands, no evidence could be obtained for significant differences in secondary structure between the cryoglobulin and noncryoglobulin immunoglobulins. Therefore, it appears most probable that the unusual circular dichroism and fluorescence spectra of the cryoimmunoglobulins reflect differences in tertiary structure between the cold-insoluble and soluble proteins.

**Temperature-Induced Conformational Change.** The effect of temperature on the intensity and position of both the circular dichroism ellipticity minimum (217–218 nm) and fluorescence emission maximum (320–340 nm) of each protein was inves-

tigated in order to explore possible low-temperature-induced conformational changes (7) (results not illustrated). Experiments were performed at sufficiently decreased concentration and in appropriate solvent systems so that the cryoimmunoglobulins maintained their solution state. With the previously noted exception of McE, no evidence was obtained for structural transitions in the cryoglobulin molecules at low temperature (7). These observations were confirmed by additional temperature studies involving laser Raman spectroscopy with high concentrations (>50 mg/ml) of McE, Pel, and Ger. The results did not support a general role for major (experimentally detectable) changes in secondary or tertiary structure of monoclonal cryoimmunoglobulins at the temperatures at which they become insoluble. The exception, protein McE, may be an instance of an immunoglobulin that displays an unusual sensitivity to low temperatures at low protein concentrations.

### CONCLUSIONS

This study has addressed three central issues regarding monoclonal cryoimmunoglobulins: (i) Can unique structural features be associated with cryoimmunoglobulins when they are compared to their cold-soluble counterparts? (ii) What is the nature of the intrinsic structural features responsible for their characteristic low-temperature insolubility? (iii) Are there significant individual differences between these proteins? With regard to the first question, it is apparent that no single unique structural feature was associated with all of the proteins. In terms of subunit molecular weights, subunit composition, sensitivity to limited enzymatic proteolysis, amino acid composition, and pI, the proteins are remarkably similar to the noncryoglobulin reference proteins. The distribution of variable region subgroups was sufficiently heterogeneous to preclude variable region-cryoglobulin correlations. When the solution conformation of these proteins is examined, however, it can be noted that half the proteins exhibit atypical circular dichroism spectra, and five of the six proteins exhibit a similar anomalous quenching of tryptophan fluorescence. Three of the five proteins that exhibit atypical fluorescence emission spectra also demonstrate anomalous gel filtration behavior, further suggestive of an atypical tertiary structure.

These studies also shed some light on the actual mechanisms of cryoprecipitation. The lack of cryoprecipitability of cryoglobulin subunits or bivalent fragments and the failure of fragments to inhibit or enhance cryoprecipitability of the parent molecules argue against classical antibody-antigen complex formation. Based on our earlier observations that certain non-cryoglobulin, monoclonal immunoglobulins exhibited cryoglobulin-like behavior in isotonic  $^2\text{H}_2\text{O}$  compared to  $^1\text{H}_2\text{O}$ , we postulated that cryoglobulin behavior may be more simply understood as a general solubility phenomenon (9). Presumably owing to an atypical conformation, the proteins interact unfavorably with solvent (maximizing intermolecular association) as the temperature is decreased. It therefore would be predicted that higher molecular weight macromolecules with correspondingly greater surface areas would more readily achieve solvent-excluding intermolecular association(s). The finding that a significantly greater percentage of IgM paraproteins manifests cryoglobulin tendencies (3-6) supports this hypothesis, as does the observation that, when the concentration dependence of cryoprecipitation of five of the six IgM and IgG proteins in this study are compared, the IgM proteins cryoprecipitate at lower concentrations than do the IgGs.

The remaining issue concerns whether or not different cryoglobulins cryoprecipitate for similar or different reasons. Differences in the concentration and temperature dependence of cryoprecipitation, as well as both qualitative and quantitative difference in the effect of solutes on cryoprecipitation, suggest that the physicochemical basis for cold-dependent insolubility of the six proteins differs. Although the fluorescence (and some circular dichroism) data argue that most of the proteins have an atypical tertiary structure, the fact the gel filtration anomaly is characteristic of only some of the proteins (with atypical fluorescence emission spectra) suggests that further classification is possible. From these data it is reasonable to conclude that cryoprecipitation can result from more than one type of conformational irregularity and that qualitatively different molecular explanations may be necessary to explain the atypical low-temperature insolubility of each cryoimmunoglobulin.

This is paper no. 7 in a series dealing with the molecular basis for cryoglobulin behavior. We are indebted to Drs. E. Breslow, R. A. Good, A. Rosenberg, and A. S. Schneider for valuable suggestions concerning this work. Drs. J. D. Capra, C. Christian, L. Reich, and B. Underdown supplied many of the cryoglobulins and noncryoglobulin reference proteins. Subclass assignments for the IgG proteins were provided by Drs. J. D. Capra and A. C. Wang. Portions of this work were submitted by C.R.M. in partial fulfillment for the requirement of doctor of philosophy awarded by Cornell University Graduate School of Medical Sciences. This research was supported by National Cancer Institute Grant CA-08748 and National Institutes of Health Grant AI-13528.

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